Reaction of the N-Terminal Methionine Residues in Cyanase with Diethylpyrocarbonate†

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ABSTRACT: Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the reaction of cyanate with bicarbonate to give ammonia and carbon dioxide. The enzyme is a decamer of identical subunits (M_r = 17 000). Previous studies have shown that modification of either the single cysteine residue or the single histidine residue in each subunit gives an active decameric derivative that dissociates reversibly to inactive dimer derivative, indicating that decameric structure is required for activity and that the SH and imidazole groups are not required for catalytic activity [Anderson, P. M., Korte, J. J., Holcomb, T. A., Cho, Y.-G., Son, C.-M., & Sung, Y.-C. (1994) *J. Biol. Chem. 269*, 15036−15045]. Here the effects of reaction of the reagent diethylpyrocarbonate (DEPC) with cyanase or mutant cyanases are reported. DEPC reacts stoichiometrically with the histidine residue and at one additional site in each subunit when the enzyme is in the inactive dimer form, preventing reactivation. DEPC reacts stoichiometrically (with the same result on reactivation) at only one site per subunit with the inactive dimer form of cyanase mutants in which the single histidine residue has been replaced by one of several different amino acids by site-directed mutagenesis; the site of the reaction was identified as the amino group of the N-terminal methionine. DEPC does not react with the histidine residue of the active decameric form of wild-type cyanase and does not affect activity of the active decameric form of wild-type or mutant cyanases. Reaction with the N-terminal amino group of methionine apparently prevents reactivation of the mutant enzymes by blocking association to decamer. However, some reactivation of the wild-type cyanase occurs when the free histidine is regenerated by treatment with hydroxylamine; a possible explanation is that the presence of the histidine residue overcomes the effect of the reaction of DEPC with the N-terminal amino group, which is consistent with previous observations that histidine plays a significant role in stabilizing the decamer and facilitating decamer formation. The rate of reaction of the dimer of cyanase with DEPC is increased 2−3-fold by the presence of 50 mM bicarbonate, suggesting that the substrate bicarbonate can bind to the inactive dimer, but that the binding constant is much higher than for the active decamer.

Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the following reaction between cyanate and bicarbonate (Taussig, 1965; Anderson, 1980; Johnson & Anderson, 1987):

$$NCO^- + H^+ + HCO_3^- \rightarrow NH_2CO_2^- + CO_2$$

Cyanase is an oligomer of identical subunits (subunit M_r = 17 000) (Anderson, 1980; Chin et al., 1983; Sung et al., 1987). The purified enzyme has been crystallized (Kyung et al., 1987), and X-ray crystallographic data have recently been collected on the native enzyme at a resolution of less than 2 Å (Otwinowski et al., 1991); these preliminary data show that the enzyme is a decamer with 5/2 symmetry, i.e., a pentamer of dimers. Equilibrium dialysis binding studies have shown a stoichiometry of one binding site per two subunits for oxalate [analog of the proposed dianion intermediate formed in the reaction between bicarbonate and cyanate (Anderson & Little, 1986)], chloride (analog of cyanate), and bicarbonate, i.e., these ligands display apparent half-site-binding (Anderson et al., 1987). The kinetic mechanism of cyanase is rapid equilibrium random with significant competitive substrate inhibition by both substrates (Anderson & Little, 1986).

Reaction of the single SH group in each subunit with certain SH reagents, such as methyl methanethiosulfonate or tetranitromethane, gives an active decameric enzyme that can be caused to dissociate reversibly to inactive dimer by lowering the ionic strength and/or lowering the temperature from 26 to 0 °C in the absence of bicarbonate (Anderson et al., 1988). Reassociation (and reactivation) is facilitated by increasing the ionic strength and/or temperature and also by the presence of substrate (bicarbonate) or substrate analogs (e.g., azide or chloride). Mutant enzymes in which either the single cysteine or histidine residue in each subunit was substituted by certain other amino acid residues are active but are considerably less stable, dissociating reversibly to inactive dimer under conditions where the native enzyme is stable (e.g., absence of bicarbonate at low ionic strength and temperature) (Anderson et al., 1988, 1994). A disulfide bond can form between the two SH groups (one SH group from each subunit) when cyanase exists as a dimer but not when the dimers are associated to give decamer; the inactive disulfide dimer cannot associate to decamer under any conditions (Anderson et al., 1994). Thus (1) the single cysteine and histidine residues in each subunit are not required for catalytic activity, (2) decameric structure is required for activity, and (3) the two SH groups in a dimer are apparently in close proximity to each other in the dissociated dimer but not when the dimer is associated to decamer (Anderson et al., 1994).

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A model accounting for the observed apparent half-site-binding and the requirement of decameric structure for activity has been proposed in which each subunit in the decamer is considered to have a single binding site accommodating either bicarbonate or cyanate and juxtapositioning of sites from two different subunits results in one intersubunit active site per dimer pair in the decamer (Anderson et al., 1994). The availability of mutants that are active but dissociate reversibly to inactive dimers provides an opportunity to carry out hybridization studies to test this model. However, active site residues that can be modified in such a way that activity is lost but the structural properties are not altered (e.g., decamer stability is retained) need to be identified before this approach can be applied. The studies reported here represent results of an investigation with the reagent DEPC[1] which were carried out as part of an effort to identify active site residues. DEPC has been widely used to specifically derivatize histidine residues in proteins; however, the reagent does react with other nucleophilic groups such as cysteinyl SH, lysyl ϵ-amino, tyrosyl hydroxyl, and N-terminal amino groups (Miles, 1977; Lundblad, 1991). The results show that DEPC reacts with both the single histidine residue and the amino group of the N-terminal methionine residue in each subunit when the enzyme exists as inactive dimer (but not when dimers are associated to active decamer) and prevents association of the dimer to decamer. The effect on activity (i.e., prevention of association to decamer) is the same when mutant cyanase in which the histidine residue is replaced by other amino acids is reacted with DEPC, indicating that the effect of DEPC on activity is due to reaction with the N-terminal α-amino group of methionine. The results are discussed in terms of the role of these two residues in cyanase function.

MATERIALS AND METHODS

Materials. Wild-type and mutant cyanase were isolated as previously described, except that all buffers contained 10 mM bicarbonate and 5 mM azide to prevent dissociation of cyanase decamers to inactive dimers (Sung et al., 1987; Anderson et al., 1994). The dimer disulfides of wild-type and mutant cyanases were prepared as previously described (Anderson et al., 1994). Proteolytic enzymes, most biochemicals, and [^{14}C]DEPC were purchased from Sigma Chemical Co.

Reactions and Analyses involving DEPC. DEPC was dissolved in ethanol or acetonitrile and added to reaction mixtures so that ethanol or acetonitrile concentration was less than 1%. DEPC was reacted with either the active decameric form of wild-type or mutant cyanases or the inactive dissociated dimer form of wild-type or mutant cyanases. The dimer form was stabilized as a disulfide dimer, which has an intersubunit disulfide bond that prevents association to decamer; the disulfide bond can be reduced by reaction with DTT, giving the native dimer, allowing association to decamer (Anderson et al., 1994). Reduction and association of disulfide dimer to decamer, resulting in regeneration of catalytic activity, after reaction with DEPC for various times was accomplished by adding an aliquot of the reaction mixture to 3 volumes of 0.2 M phosphate buffer, pH 7.3, containing 0.05−0.1 M DTT, 0.08−0.12 M bicarbonate, and 0.04 M imidazole or histidine (the latter two compounds react with remaining DEPC) at 26 °C; after incubation for at least 20 min, a small aliquot was assayed (Anderson et al., 1994). Bound radioactivity resulting from reaction of [^{14}C]DEPC with cyanase was determined by precipitating the protein with 10% TCA, followed by centrifugation, washing with 10% TCA, suspending the precipitated protein pellet in 0.1 N NaOH, and counting with a scintillation counter. This is similar to a procedure previously described (Gasparini, 1991) and gave results similar to those obtained when centrifugal gel filtration chromatography was used to remove unbound [^{14}C]DEPC.

Peptides obtained from proteolytic digests were separated by HPLC using a Beckman Ultrapore RPMC 4.6 × 7.5-cm C-8 column with a 0−60% (1 mL/min, 90 min) or 19.5−20.5% (1 mL/min, 2 h) acetonitrile in 0.1 M trifluoroacetic acid gradient. Amino acid analysis was carried out as previously described, except 0.1% thioglycolate was present during acid hydrolysis to minimize oxidation of methionine (Anderson et al., 1988).

Other Methods. Cyanase activity and kinetic analyses were carried out as previously described (Anderson & Little, 1986; Anderson et al., 1987). The molecular size of cyanase derivatives (dimer versus decamer) was determined by high-performance gel filtration chromatography and sucrose density gradient centrifugation as previously described (Anderson et al., 1994). Cyanase concentration was measured quantitatively by its A_{280}, adjusted appropriately for the change in extinction coefficient when the histidine residue was replaced by a tyrosine residue (Anderson, 1980).

RESULTS

Reaction of Wild-Type Cyanase with DEPC. The single histidine residue of wild-type decameric cyanase does not react with DEPC, even at DEPC concentrations as high as 20 mM; there is no increase in A_{242} characteristic of the reaction of histidine with DEPC (Miles, 1977) and catalytic activity is not affected (data not shown). However, the histidine residue of the inactive disulfide dimer derivative of cyanase prepared from decameric cyanase does react with a low molar excess (5×) of DEPC, resulting in an increase in A_{242} consistent with reaction of one histidine residue per subunit [using an extinction coefficient of 3200 M^{-1} cm^{-1} (Lundblad, 1991)]; reaction of the disulfide dimer with DEPC also results in loss of activity (i.e., decreased reactivation of the disulfide dimer). As shown in Figure 1, although the increase in A_{242} qualitatively appears to be associated with loss in activity, the time required for half-maximal change is less for the increase in A_{242} than for the loss in activity (2 versus 4 min, respectively). The maximum loss in activity (82% in the experiment described in Figure 1) varied from one experiment to another under these conditions (5× molar excess of DEPC), but all activity could be inhibited by using a 10× molar excess of DEPC (see Figure 2). These effects were partially reversed by addition of hydroxylamine, which specifically regenerates the unmodified histidine residue from the product formed by reaction of histidine with DEPC (Miles, 1977); as noted in Figure 1, after a 20-min reaction time 60% of the initial catalytic activity was regenerated and there was a corresponding decrease in A_{242}. These results implicate histidine as at least one site of reaction of DEPC with cyanase disulfide dimer. There was no change in A_{278},

[1] Abbreviations: DEPC, diethylpyrocarbonate; DTT, dithiothreitol; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography.

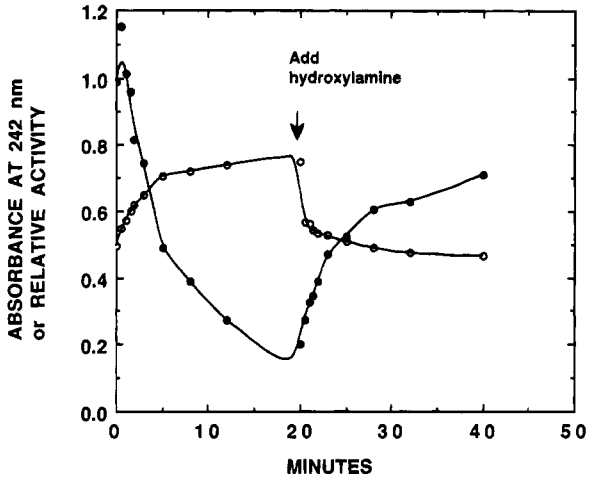

FIGURE 1: Time course of reaction of DEPC with the disulfide dimer of native cyanase. The reaction mixture contained 1 mg/mL (0.06 mM with respect to subunit concentration) disulfide dimer of cyanase and 0.3 mM DEPC in 0.01 M phosphate buffer, pH 7.3. The initial volume was 1.0 mL. At the indicated times a 50-mL aliquot was removed and subjected to reactivation and assay of activity as described under Materials and Methods. The A_{242} was measured at the same times. The specific activity of the reactivated cyanase at time zero was 198 μmol/(min·mg). At 20 min, 0.25 mL of 0.8 M NH_2OH was added to the remaining 0.5 mL of reaction mixture, as indicated. A_{242} (○); relative activity (●).

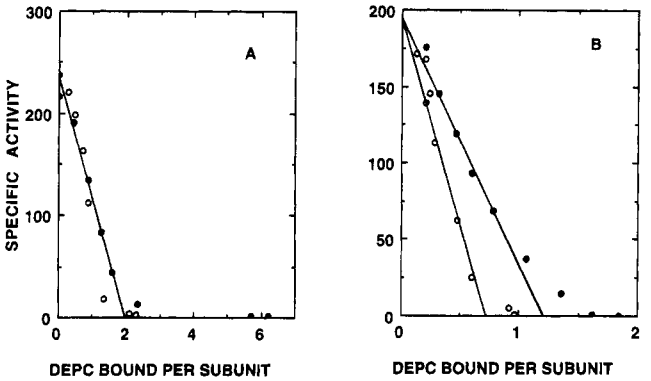

FIGURE 2: Stoichiometry of reaction of [^{14}C]DEPC with disulfide dimer of wild-type cyanase and H113Y mutant. (A) The initial reaction mixture contained wild-type cyanase disulfide dimer (1 mg/mL, 0.06 mM with respect to subunit concentration), 0.01 M phosphate buffer, pH 7.3, and 0.3 mM [^{14}C]DEPC (4×10^5 cpm) in the presence (●) or absence (○) of 60 mM sodium bicarbonate. At 0, 0.5, 1, 2, 3, and 10 min the specific activity [μmol/(min·mg)] of reactivated enzyme and the [^{14}C]DEPC covalently bound per subunit were determined as described under Materials and Methods. At 10 min the concentration of [^{14}C]DEPC in the remaining reaction mixture was increased to 0.9 mM, and the same analyses were made at 15 and 20 min. (B) Same as panel A, except H113Y mutant cyanase disulfide dimer was used instead of wild-type cyanase disulfide dimer, the concentration of [^{14}C]DEPC was not increased at 10 min, and analysis was carried out at 0.3, 0.6, 1, 1.5, 2, 3, 5, 10, and 15 min.

indicating that tyrosine residues do not react with DEPC (Miles, 1977; Lundblad, 1991).

Although essentially the same final results are obtained (see Figure 2), the rate of reaction of the disulfide dimer is increased 2−3-fold by the presence of bicarbonate. The effect is concentration dependent, with a half-maximal effect at about 15 mM; when present, 60 mM bicarbonate was employed in these studies (data not shown).

The stoichiometry of reaction of [^{14}C]DEPC with wild-type cyanase disulfide dimer is shown in Figure 2A. These

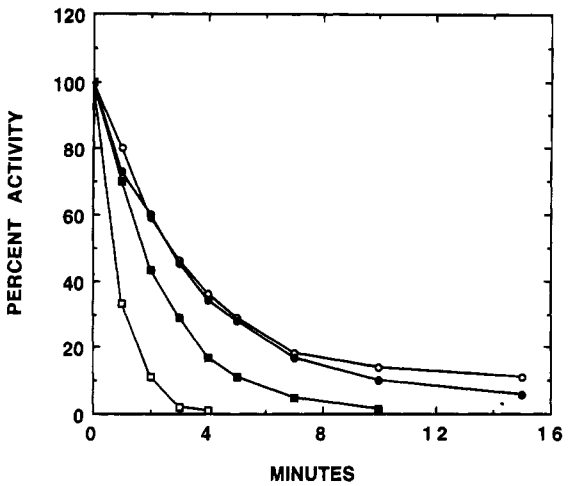

FIGURE 3: Inhibition of different histidine mutant cyanase disulfide dimers by DEPC. The reaction mixtures were essentially as described in the legend to Figure 2 (bicarbonate present), except the disulfide dimers were prepared from H113Y (□), H113N (■), H113G (●), and H113V (○) mutants. A 50−μL aliquot was removed at the indicated times and the activity determined as described under Materials and Methods (activity is expressed as percent of the initial activity).

results indicate that loss in activity correlates with reaction at two sites, one of which would be the single histidine residue. This result, together with the observation above that reaction with the histidine residue appears to be faster than loss in activity and the observation that modification of the histidine residue by site-directed mutagenesis does not inactivate the enzyme (Anderson et al., 1994), suggests that the observed inhibition of activity by DEPC may be due to reaction at the non-histidine site. The presence of bicarbonate does not affect the relationship between stoichiometry and activity, but up to four additional sites react when the DEPC concentration is increased if bicarbonate is present.

Reaction of Histidine Mutant Cyanases with DEPC. As observed with wild-type cyanase, the activity of the decameric form of the histidine mutant cyanases is not affected by DEPC. However, the activity (i.e., decreased reactivation of the disulfide dimer) of the disulfide dimers is inhibited by reaction with a low molar excess (5×) of DEPC, as shown in Figure 3. Interestingly, the rate of loss in activity is highly dependent on which amino acid replaces the histidine residue, with tyrosine resulting in the most reactive mutant and glycine and valine the least. Complete inhibition of the tyrosine and asparagine mutant disulfide dimers can be attained with only 2× molar excess of DEPC. In contrast to the wild-type enzyme, inhibition cannot be reversed by reaction with NH_2OH, suggesting that the site of reaction is with a primary amine (Miles, 1977). The stoichiometry of reaction of the mutant disulfide dimer of H113Y is shown in Figure 2B. Loss in activity correlates with reaction of DEPC at a single site. This would be consistent with the loss of one of the two reactive sites due to the absence of the single histidine residue. As observed with the disulfide dimer of wild-type cyanase, the presence of bicarbonate results in reaction of DEPC at additional sites. Reaction with DEPC prevents reassociation to decamer even under the most optimal conditions (determined as described under Materials and Methods in the presence of 0.15 M potassium phosphate, pH 7.3, and 0.05 M sodium bicarbonate at 26 °C; [see Anderson et al. (1988, 1994)]. Reaction of DEPC with

H113Y is not accompanied by a change in either A_{242} or A_{278}.

Site of Reaction of DEPC with Histidine Mutant Cyanase. The site of reaction of DEPC that affects activity of the histidine mutant cyanase disulfide dimers was identified as follows. The disulfide dimer of H113Y (1 mg/mL, 0.06 mM with respect to subunit concentration) was reacted with [^{14}C]-DEPC (0.3 mM, 9×10^5 cpm) in 0.01 M phosphate buffer, pH 7.3, for 6 min (30% of initial activity remaining; the slower rate compared to that described in Figure 2 reflects absence of bicarbonate; 100 000 cpm radioactivity bound to the enzyme). Protein was removed from the reaction mixture (0.5 mL) by centrifugation through a 5-mL Sephadex G-25 gel filtration column equilibrated with 0.05 M Tris, pH 8.0, and the derivatized protein was reacted 4 h at 37 °C with 0.2 mg of TPCK-trypsin. The resulting peptides were separated by HPLC as described in the Materials and Methods section (0−60% acetonitrile gradient). Seventy percent of the total radioactivity applied to the column was associated with one UV-absorbing peak (33 min); the remaining radioactivity was associated with 7 other minor peaks eluting at 14, 28, 42, 45, 51, 56, and 62 min. A major UV-absorbing peak at 33 min was also present in the control (absence of DEPC), indicating that the [^{14}C]peptide was not pure. The reaction of the disulfide dimer with [^{14}C]DEPC was repeated again in the same way, except [^{14}C]DEPC was 0.6 mM, 0.06 M bicarbonate was present, and the volume was 5.0 mL, resulting in isolation of a larger quantity of the [^{14}C]peptide eluting at 33 min. The isolated [^{14}C]peptide (from 5 mg of disulfide dimer) was reacted with leucine aminopeptidase (25 μg in 3 mL 0.1 M NH_4HCO_3, pH 8.5, 5 mM $MgCl_2$), the reaction was monitored by following the appearance of a new UV-absorbing, nonradioactive peak eluting at 11 min (19.5−20.5% acetonitrile gradient), and additional leucine aminopeptidase was added at 2-h intervals until reaction appeared complete after 6 h. The elution position of the radioactive peptide did not change (25 min in this different gradient), suggesting that the N-terminal residue was resistant to leucine aminopeptidase activity, perhaps due to reaction of the amino group of the N-terminal methionine with [^{14}C]DEPC. This was confirmed in two ways. First, the amino acid composition (relative ratios) of the [^{14}C]peptide was found to be Asx (0.85), Glx (2.1), Ser (0.9), Arg (1.2), Met (1.1), and Ile (2.0), which identifies it as the N-terminal peptide of cyanase that would be obtained by proteolysis with trypsin (Met-Ile-Gln-Ser-Gln-Ile-Asn-Arg). Secondly, the [^{14}C]peptide was treated with a broad-specificity protease (Sigma P-5147) (3 units in 3 mL of 0.1 M NH_4HCO_3, pH 8.5, containing 5 mM $MgCl_2$) for 2 h at 26 °C. The pH was adjusted to pH 1 with HCl, and virtually all of the radioactivity was extracted into ethylacetate; the extracted radioactivity migrated to the anode at pH 7.0 during paper electrophoresis, and acid hydrolysis followed by amino acid analysis revealed that only the amino acid methionine was present. These results provide evidence that the major site of reaction of [^{14}C]DEPC with the disulfide dimer associated with loss in activity is with the amino group of the N-terminal methionine.

DISCUSSION

The results described here show that DEPC reacts stoichiometrically with the amino group of the N-terminal methionine residue in cyanase when it exists as dimer but not when it exists as decamer and that reaction prevents association of the dimer to decamer. This suggests that the N-terminal residue is important for the structural integrity of the decamer; an additional role in catalysis for the N-terminal amino group, either protonated or uncharged, is also a possibility. The fact that DEPC does not react with the histidine residue of wild-type cyanase or inhibit the activity of wild-type or mutant cyanases unless the enzyme is dissociated to dimer (stabilized as the disulfide dimer) indicates that the histidine and N-terminal methionine residues are probably associated with the interfaces between dimers in the decamer.

Previous studies have shown that although the single histidine residue in each subunit of cyanase is not required for activity, the histidine residue (1) is required for stability of the decamer, (2) appears to facilitate association of inactive dimer to active decamer, and (3) contributes to the catalytic properties of the decamer (Anderson et al., 1994). The results described here are consistent with these observations. Thus, although DEPC reacts with the histidine residue in the dimer, the effect on activity is apparently due to reaction with the N-terminal methionine residue. However, these results do indicate that the single histidine residue probably influences the properties of the free dimer (stabilized as the disulfide dimer), since the rate of inhibition resulting from reaction of DEPC with N-terminal methionine in the disulfide dimer varies significantly depending on which amino acid replaces histidine in the mutant enzymes. The effect of the different substitutions correlates with the effect of the substitutions on decamer stability, i.e., the substitution which is the most destabilizing corresponds to the highest rate of inhibition by DEPC (Anderson et al., 1994). In addition, the role of histidine in decamer stability and/or facilitation of association of dimer to decamer suggested by previous studies (Anderson et al., 1994) is further emphasized by the observation here that inhibition of wild-type cyanase by reaction with DEPC is partially reversed by reaction with hydroxylamine. A possible explanation is that the presence of the unmodified histidine residue (after reaction with hydroxylamine) overcomes the effect of modification of the N-terminal methionine by DEPC in preventing association to active decamer. If this is correct, then it is likely that the function of the amino-terminal residue is related only to decamer formation or stabilization of decamer and not to catalytic activity.

The accelerating effect of bicarbonate on the rate of the reaction with DEPC suggests that bicarbonate can bind to the dimer form of the enzyme, but that the binding constant is much higher than for the decamer form of cyanase. Previous binding studies indicating that substrates or substrate analogs do not bind to the dimer would not have detected binding that required such a high concentration of bicarbonate (Anderson et al., 1987, 1994). This observation is consistent with the conclusion that dissociation of decamer to dimers is accompanied by significant conformational changes in the subunits of the dimer, which in this case is manifested by a marked decrease in the affinity for bicarbonate (Anderson et al., 1994). The effects of bicarbonate [i.e., the presence of bicarbonate (1) increases the rate of reaction of the disulfide dimers with DEPC at the site that affects activity and (2) facilitates reaction at other nonspecific sites when

the concentration of DEPC is increased] also suggests that binding of substrate results in a conformational change in the dimer. With respect to the model cited in the introduction in which the active site is formed by juxtapositioning of two identical sites from two different subunits in the decamer, this may reflect substrate-induced asymmetry (as opposed to association-induced asymmetry) for establishing specificity for either cyanate or bicarbonate (Anderson et al., 1994).

These results emphasize the importance of establishing the stoichiometry of reaction with [^{14}C]DEPC and correlation with the number of histidine residues modified and critically eliminating the possibility of reaction with other residues before concluding that an effect on activity by reaction with DEPC is due to modification of a histidine residue.

REFERENCES

Anderson, P. M. (1980) *Biochemistry 19*, 2882−2887.

Anderson, P. M., & Little, R. M. (1986) *Biochemistry 25*, 1621−1626.

Anderson, P. M., Johnson, W. V., Endrizzi, J. A., Little, R. M., & Korte, J. J. (1987) *Biochemistry 26*, 3938−3943.

Anderson, P. M., Johnson, W. V., Korte, J. J., Xiong, X., Sung, Y.-C., & Fuchs, J. A. (1988) *J. Biol. Chem.* 263, 5674−5680.

Anderson, P. M., Korte, J. J., Holcomb, T. A., Cho, Y.-G., Son, C.-M., & Sung, Y.-C. (1994) *J. Biol. Chem. 269*, 15036−15045.

Chin, C. C. Q., Anderson, P. M., & Wold, F. (1983) *J. Biol. Chem. 258*, 276−282.

Gasparini, S., Vincendon, P., Eriani, G., Gangloff, J., Boulanger, Y., Reinbolt, J., & Kern, D. (1991) *Biochemistry 30*, 4284−4289.

Iyer, K. S., & Klee, W. A. (1973) *J. Biol. Chem. 248*, 707−710.

Johnson, W. V., & Anderson, P. M. (1987) *J. Biol. Chem. 262*, 9021−9025.

Kyung, H. K., Honzatko, R. B., Little, R. M., & Anderson, P. M. (1987) *J. Mol. Biol. 198*, 137−138.

Little, R. M., & Anderson, P. M. (1987) *J. Biol. Chem. 262*, 10120−10126.

Lundblad, R. L. (1991) *Chemical Reagents for Protein Modification,* 2nd ed., Chapter 9, pp 105−128, CRC Press, Boca Raton, FL.

Miles, E. W. (1977) *Methods Enzymol. 47*, 431−442.

Otwinowski, Z., Anderson, P. M., Fuchs, J. A., Sigler, P. B., & Joachimiak, A. (1991) *Proceedings of the IV International Meeting on Crystallization of Biological Macromolecules*, August, Freiburg, Germany.

Sung, Y.-C., Anderson, P. M., & Fuchs, J. A. (1987) *J. Bacteriol. 169*, 5224−5230.

Taussig, A. (1965) *Can. J. Biochem. 43*, 1063−1069.